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Molecular Therapeutics of HBV

Ruian Xu[1,2,3,*], Kexia Cai[1], Dexian Zheng[3], Hong Ma[3], Sue Xu[2] and Sheung-tat Fan[2]

[1]*Gene Therapy Laboratory, IMB,* [2]*Center for the Study of Liver Diseases, The University of Hong Kong, Hong Kong and* [3]*School of Basic Medicine, Peking Union Medical College, Beijing, China*

Abstract: The hepatitis B virus (HBV) infection is a public health problem worldwide, particularly in East Asia. The current therapy of HBV infection is mostly based on chemical agents and cytokines that have been shown to provide limited efficacy and are also toxic to the human body.

Gene therapy is a new therapeutic strategy against HBV infection, involving the transmission of gene drugs into liver cells by specific delivery systems and methods. Although this new anti-HBV infection technique is under active investigation, various promising anti-HBV viral gene drugs have been developed for gene therapy, including antisense RNA and DNA, hammerhead ribozymes, dominant negative HBV core mutants, single chain antibody, co-nuclease fusion protein, and antigen. In order to optimize their antiviral effects and/or enhance anti-HBV immunity, various novel gene delivery systems have also been developed to (specifically) deliver such DNA constructs into liver cells: some of them are viral vectors, such as adenoviral vectors, retroviral vectors and poxviral vectors, and even hepatitis B viral for its hepatocellular specificity. Others are non-viral vectors, in which naked DNA and liposomes are frequently used for DNA vaccine or nucleotide analogs for inhibiting HBV DNA polymerase.

This review addresses various aspects of gene therapy for HBV infection, including gene drugs, delivery methods, animal model, and liver transplantation with combination therapy. It also discusses the problems that remain to be solved.

1. INTRODUCTION

Despite the availability of efficient vaccines for protecting previously unexposed individuals, hepatitis B virus (HBV) infection remains a major cause of morbidity and mortality worldwide. There is significant geographic variation in infection rates, but it is estimated that 350 million people worldwide have chronic HBV infection [Lee, 1997]. In Southeast Asia, Africa and China, more than 50% of the population is infected, and 8% to 15% have become chronically infected. Chronic HBV infection is the cause of up to 50% of cirrhosis cases and 70%-90% of hepatocellular carcinoma (HCC) in these regions [Lok, 1992; Fattovich, 1998]. Between 250,000 and 300,000 new HBV infections occur annually, and 4,000 to 5,000 persons die annually from cirrhosis or liver cancer in the United States. Neonatal HBV infection nearly always results in chronic HBV infection. Pre-existing immunosuppression also increases the risk of chronic infection. Because the prevalence of infection is largely determined by a feedback mechanism, and the carriers are the most influential, predictive quantity, reduction in carrier prevalence via therapeutic treatment can be a means of controlling the incidence of HBV quickly relative to the effect of immunization and behavior modification [Medley *et al*., 2001].

Chronic hepatitis B is the result of a continuing attack on infected cells by the host immune system, which is not vigorous enough to eradicate all the infected hepatocytes [Chisari, 1995]. Therefore, two main non-exclusive strategies can be envisaged to eradicate viral infection: inhibition of viral replication and/or nonspecific inhibition of viral replication combined with enhancement of immune response [Zoulim, 1999]. Currently, the only therapy for chronic hepatitis B with a lasting beneficial effect is the systemic treatment with interferon (IFN-α having dual properties, in that it inhibits viral replication and also stimulates cell mediation immunity to HBV by mechanisms that have not been fully delineated. The sustained response is achieved in only one third of patients [Hoofnagle and Bisceglie, 1997]. Any patients with chronic hepatitis B and markers of active viral replication, elevated ALT level and chronic hepatitis on liver biopsy are potential candidates for IFN-α therapy. HBV genotype C, compared to genotype B, is associated with a higher frequency of core promoter mutation, and a lower response rate to interferon alpha therapy [Kao *et al*., 2000]. In addition to its severe side-effects [Hoofnagle and Bisceglie, 1997; Main *et al*., 1998], IFN-α exhibits a short half-life in blood after parenteral protein administration [Heremans *et al*., 1980], which limits its performance, as it is unable to make sufficient or sustained deliveries of the protein into the liver. Recently, nucleoside analogs have provided a therapeutic alternative, leading to a rapid decrease in serum HBV DNA levels and to a histologic improvement in the treatment of liver disease [Lai *et al*., 1998; Urban *et al*., 2001]. Clinical data show that lamivudine is an effective treatment for a wide range of patients with chronic hepatitis B, whose advantages include lower cost, peroral administration and a higher patient tolerance than IFN [Yao, 2000], however, short-term treatment leads to a rapid relapse of the disease, and long-term treatment often results in the developing of drug toxicity [Lee, 1995] or the selection of resistant viral variants

*Address correspondence to this author at the Gene Therapy Laboratory, IMB, Center for the Study of liver Diseases, The University of Hong Kong, Hong Kong and School of Basic Medicine, Peking Union Medical College, Beijing, China; Tel: 00852-2299-0757, Fax: 00852-2817-1006, E-mail rxua@hkucc.hku.hk

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[Zoulim and Trepo, 1998]. These outcomes emphasize the need for novel therapeutic approaches [Hoofnagle and Bisceglie, 1997]. In fact, the more advances in our understanding of the mechanisms underlying virus life histories, the more opportunities for the rational design of molecules that could specifically block viral infection, replication and maturation. Moreover, a combination of therapies with different mechanisms of actions may be most effective and prevent the selection of resistant viral strains. Genetic therapy may be one such therapeutic approach, as many transgenes have been shown to inhibit HBV *in vitro* or *in vivo*.

2. MOLECULAR DRUGS

2.1 Antiviral Strategies

2.1.1 Anti-Sense Nucleotide

Besides the study of cellular and viral gene functions, active research on antisense nucleotides is also underway, for their potential to interfere with viral gene expression as antiviral agents. The processes of replication, transcription, and translation of HBV can be blocked by antisense molecules specifically binding to target genes to inhibit viral actions. Previous studies have shown that a number of antisense oligonucleotides against HBV mRNA are able to inhibit viral gene expression *in vitro*. Many of these effective molecules, whether chemically synthesized or endogenously expressed, targeted almost all the specific functional sequences of viral genes. In the cell culture system, antisense DNA directed to HBV polyadenylation signal [Nakazono *et al.*, 1996; Wu *et al.*, 1992], 5'-upstream sequences [Nakazono *et al.*, 1996] and X gene [Feng *et al.*, 1997], and antisense RNA complimentary to preS/S [Tung and Bowen, 1998; Ji *et al.*, 1997; Wu and Gerber 1997], preC/C [Ji *et al.*, 1997; Soni *et al.*, 1998], HBV X and HBV P [Wu *et al.*, 2001], are particularly effective. Without an identical model, it is not certain which target is resultantly more effective. Korba and Gerin examined the ability of 56 different single-stranded oligodeoxyribonucleotides (14-23 nucleotides in length), which target several HBV-specific functions, to inhibit HBV replication in the human hepatoblastoma cell line 2.2.15. The oligomers directed against the HBV encapsidation signal/structure (epsilon) showed the most effective antiviral efficacy against HBV [Korba and Gerin, 1995]. Dual-target antisense RNA, expressed by retroviral vector in HepG2.2.15 cells, also exhibited higher inhibitory effect than its single-target counterparts [Wu *et al.*, 2001]. *In vivo*, an antisense oligodeoxynucleotide directed against the 5'-region of the preS gene of DHBV inhibited viral replication and gene expression in Peking ducks [Offensperger *et al.*, 1998]. Furthermore, poly-DNP-RNA with antisense RNA targeted against the DHBV polymerase gene could completely inhibit duck viremia, and thus viral DNA disappeared [Xin *et al.*, 1998]. Another antisense oligomer, complementary to the cap site of the SP II promoter of HBV mRNA, also produced an effective inhibitory effect, after injected into athymic nude mice producing HBV markers [Yao *et al.*, 1996]. Putlitz and Wands compared sense and antisense RNAs on HBV replication, and found that both inhibited HBV replication, but only sense sequence inhibited HBsAg secretion [Putlitz *et al.*, 1999]. This demonstrated that both sense and antisense base strategies could be successfully used to inhibit viral replication [Ding *et al.*, 1998].

A new concept of antisense is covalently linked to Ribonuclease H to obtain a direct specific cleavage event. It has been tested in tube to cleave HBV mRNA with the sequence recognizing ability of oligonucleotide specific for the DR1 region linked with RNase H [Walton *et al.*, 2001].

2.1.2 Ribozyme

Ribozymes act as RNA-cleaving RNA molecules that can catalyze the cleavage and inactivation of other cellular and viral RNA molecules with a specific nucleotide sequence. Their success *in vitro* is unquestioned, and the use of ribozymes, especially small catalytic RNAs, in antiviral gene therapy is also being actively pursued [Welch et al, 1998; Wong-Staal *et al.*, 1998; Macpherson *et al.*, 1999; Amado *et al.*, 1999]. The hammerhead ribozyme and the hairpin ribozyme have been designed to inhibit HBV gene expression, and have been analyzed in a cell culture system. Presently, a chemically modified ribozyme, targeting HBV mRNA, is at the clinical development stage. The main challenge for the use of ribozyme is the search for accessible target sites on a substrate RNA. A combinatorial screening method has been used to identify catalytically active hairpin ribozymes that mediate intracellular antiviral effects on HBV [Zu *et al.*, 1999]. Several hammerhead ribozymes also have been used with varying success to inactivate HBV RNA. The targeted sequences include poly (A) signal sequence [Feng *et al.*, 2001], the tail region of the HBV core protein [Feng *et al.*, 2001], and dual sites in HBc RNA [Li *et al.*, 2000]. Three hairpin ribozymes have been designed to target the pgRNA and specific mRNAs encoding the HBsAg, the polymerase and the X protein, and cotransfected into HuH-7 HCC cell together with small amount of an HTD of HBV in transfected human hepatocellular carcinoma (HCC) cells. As a result, the virus particles-associated HBV levels were reduced up to 83% [Welch *et al.*, 1997]. HBx RNA is the most plausible target for the ribozyme to block the HBV replication because the sequence of the smallest X transcription is fully included in the 3' sequence of all HBV transcription. Using ribozyme-encoding vectors to transfect liver cells, two research groups found that hammerhead ribozyme targeting to HBx sequence cleaved the substrate in a catalytic manner [Passman *et al.*, 2000; Kin *et al.*, 1999], however, Weinberg et al suggested that an antisense mechanism without substrate cleavage might be the dominant intracellular effect [Weinberg *et al.*, 2000].

Previous unsuccessful attempts [von Weizsacker *et al.*, 1992; Beck *et al.*, 1995] at using hammerhead ribozymes for the intracellular inhibition of HBV revealed that activity-selected ribozymes are likely to be more effective than sequence-selected ones, with respect to intracellular inhibitory effects. Extra sequences endogenously coexpressed with ribozyme may have an effect on the folding of the RNAs, and thus affect ribozyme formation and its accessibility. A recent report showed that the insertion of trans-ribozyme between two cis-ribozyme sequences led to the removal of the extra sequences and the abolishment of any cis-inhibitory effect from the non-ribozyme sequence [Feng *et al.*, 2001]. This argues that the level of the ribozyme expression is correlated with its inhibition efficiency in

cultured cells, which was found in a previous study [Kin *et al.*, 1999]. And this outcome indicates that a more effective delivery system is required in anti-HBV gene therapy. According to a published work on HIV, Rev-binding RNAs efficiently block HIV-1 gene expression, whereas other antisense RNA and ribozymes have little or no effect when expressed in the same cassettes. This observation demonstrates that different promoters should be chosen when expression cassettes are constructed to express different antisense RNAs and ribozymes in order to transcribe them efficiently, stabilize them against rapid degradation, fold them correctly, and deliver them directly to appropriate part of a cell [Good *et al.*, 1997].

The efficiency of ribozyme action in the complex intracellular environment is difficult to predict. Development of therapeutic sequences is often guided by the empirical assessment of intracellular functional inhibition of a target [Weinberg *et al.*, 2000], and thus is not always reliable. Plasmids containing intact HBV sequences or a modification in which the preS2/S region was replaced by DNA encoding enhanced green fluorescent protein (EGFP) were used to test ribozyme action in transfected cells. The measurement of EGFP expression is convenient to assess ribozyme action *in situ* [Passman *et al.*, 2000].

2.1.3 Triplex Forming Oligodeoxynucleotide

Unlike an antisense oligonucleotide, a triplex forming oligodeoxynucleotide (TFO) acts directly on the gene by binding to duplex DNA in a stable, sequence-specific manner. This oligonucleotide-directed triplex DNA formation has been shown to inhibit transcription factor binding to purine-rich motifs, and the TFOs have been used in this way to block transcription of various genes *in vitro* and in intact cells [McGuffie *et al.*, 2000; Jendis *et al.*, 1998]. Gao *et al.* designed a TFO that can form triplex with SP1 sites in HBV core promoter. When transfected into HepG 2.2.15 cells by packing with liposome, the synthesized TFO can effectively inhibit replication of HBV [Gao *et al.*, 2001]. The blocking of viral replication at transcriptional level with TFOs is a very promising molecular approach, although results in HBV infection are rare.

2.1.4 Aptamer

Many aptamers are designed to block protein functions. Aptamer, a small nucleotide, can bind to its ligand (protein, ion, antibiotic, etc.) with high affinity and high specificity. Their binding is due to their 3-D conformation. There is no sequence complementation between an aptamer and its ligand. Recently eight peptide aptamers were isolated from a randomized expression library, which specifically bound to the HBV core protein under intracellular conditions. One of them inhibited HBV replication by blocking viral capsid formation. This provides a new basis for the development of therapeutic molecules with specific antiviral potential against HBV infection [Butz *et al.*, 2001]. Accumulated findings about interaction between cellular proteins and specific sequences of HBV gene may give some hints for application of this new approach. Since interaction between nuclear receptors and the nuclear receptor response elements (NRREs) present in the HBV genome may play critical roles in regulating its transcription and replication during HBV infection of hepatocyes [Yu *et al.*, 2001], an aptamer can be designed to express in nucleus to compete for binding to the nuclear reporter proteins, and may result in anti-HBV infection. Similarly, aptamers binding to YY1, a transcription factor, may prevent HBV genome integrating into the cellular DNA, according to the report that integrated hepatitis B virus DNA preserves the binding sequence of YY1 at the virus-cell junction [Nakanishi-Natsui *et al.*, 2000]. For HBV pregenomic (pg) RNA to be encapsidated, its 5' end is folded into a stem-loop structure, that is the encapsidation signal (epsilon), which is involved in the activation of polymerase [Kramvis and Kew, 1998]. This offers the possibility of inactivating HBV polymerase protein by an aptamer, probably a more attractive and effective approach.

2.1.5 Nucleoside Analog

Nucleoside analog replaces naturally occurring nucleosides such as adenosine, guanosine, cytidine, and thymidine and uridine, and causes DNA chain termination. Using the avian HBV model system, Urban *et al.* obtained new insights into the catalytic mechanism of HBV reverse transcriptases (RT). It was shown that pyrophosphate (PPi)-dependent RT activities were able to efficiently remove newly incorporated nucleotides and certain antiviral drugs even under low, cytoplasmic concentrations of PPi. These activities operating during viral replication could potentially undermine the efficacy of some drugs. Analysis of chain-terminated DNA revealed that the potent anti-HBV drug lamivudine (3TC) was difficult to remove by pyrophosphorolysis, in contrast to ineffective chain terminators such as ddC. Therefore, it was suggested that HBV-RT pyrophosphorolysis activity may be a novel determinant of antiviral drug efficacy, and could serve as a target for future antiviral drug therapy [Urban *et al.*, 2001].

Nucleoside analog drugs suppress the replication but do not eradicate the HBV. As a result, stopping the medication may lead to a relapse of HBV. Lamivudine therapy induces improvements in chronic hepatitis B in a high proportion of patients, but prolonged therapy is limited by the development of viral resistance [Zoulim and Trepo, 1998]. Clinical data showed that long-term therapy with lamivudine resulted in sustained improvements in virologic, biochemical, and histologic features of disease in most patients with HBeAg-negative chronic hepatitis B and in the subgroup of HBeAg-positive patients with high serum transaminase levels. A high rate of resistance limited efficacy, particularly in patients who remained HBeAg positive on therapy [Lau *et al.*, 2000]. The HBV-specific CTL response before and during lamivudine therapy was studied longitudinally in 6 HLA-A2-positive patients with HBeAg+ chronic hepatitis B. This study shows that lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B [Boni *et al.*, 2001]. With the use of lamivudine, induced antiviral immune responses and consequent viral elimination have been observed in chronic hepatitis B patients who received six monthly intradermal vaccinations with HBsAg or together with daily Interleukin-2 (IL-2) s.c. [Dahmen *et al.*, 2002]. These data demonstrate that a cure for chronic HBV infection may be achieved by treatment with lamivudine in combination with immune therapies.

The FDA approved Lamivudine, a nucleoside analog, in 1998 for the treatment of chronic HBV infection. Nucleoside

analogs of this type directly block HBV polymerase-reverse transcriptase and inhibit viral replication. Treatment with lamivudine at a dose of 100 mg given orally once daily results in a rapid decrease in the HBV DNA level and marked improvement in measures of liver injury [Mailliard and Gollan, 2003]. The major problem with lamivudine monotherapy has been the emergence of drug-resistant HBV polymerase (YMDD) mutants. As a result, long-term use of lamivudine in other settings remains controversial [Perrillo, 2002]. Newer nucleoside analogs are being extensively investigated by studies *in vivo* and *in vitro*. According to *in vitro* studies, the resistance of HBV DNA polymerase mutants M552I, M552V to lamivudine triphosphate with inhibition constants (Ki) increased compared with that of wild-type HBV DNA polymerase. Encouragingly, these mutants remained sensitive to adefovir diphosphate, with the inhibition constants increasing 1.3 times and 2.2 times [Xiong *et al.*, 1998]. In pre-clinical and phase 2 studies of patients with HbeAg-positive chronic hepatitis B, 48 weeks of 10 mg or 30 mg of adefovir dipivoxil per day resulted in histologic liver improvement, reduced serum HBV DNA and alanine aminotransferase levels, and increased rates of HbeAg sero-convension. The 10 mg dose has a favorable risk-benefit profile for long-term treatment. No adefovir associated resistance mutations were identified in the HBV DNA polymerase gene [Marcellin *et al.*, 2003]. Perrillo *et al.* [2000] also demonstrated that adefovir dipivoxil was effective against lamivudine-resistant hepatitis B virus (HBV). Five patients with chronic HBV infection developed resistance to lamivudine after 9 to 19 months of treatment, and were then treated with adefovir dipivoxil in a dose of 5 to 30 mg daily. Two to 4 log (10) reductions in HBV-DNA levels were observed in 4 cases, and the fifth patient becomes negative by quantitative polymerase chain reaction after retransplantation in conjunction with hepatitis B immunoglobulin (HBIg). Combination therapy with two or three nucleotide analogs will become one of the main treatments of chronic hepatitic B in future.

2.1.6 Peptide

Peptide may be used as a therapeutic antigen by interfering with the interaction between HBV particles and the cell surface or the viral replication and maturation. The studies on components associated with the internalization of HBV particles may prove to be very useful to protect cells from infection, and consequently block viral replication. The existence of a fusogenic sequence was predicted in the junction area of the PreS2- and S-domain of the hepatitis-B virus surface antigens. Evidence has been produced that the sequence 7-18 of the hepatitis B S domain [Berting *et al.*, 2000], and motif amino acids 41 and 52 of PreS2 [Oess and Hildt, 2000] mediated cell-permeability. They may initiate the first step of viral entry. Correspondingly, the domain of the cellular reporter, carboxypeptidase D [Tong 1999], was identified, which may play a role in the binding and presentation of proteins or peptide substrates [Aloy *et al.*, 2001]. These efforts have increased the chances for the design of a peptide to block viral infectivity. Recently, a myristoylated Pre-S peptide was used in DHBV model. Though lacking in the essential part of the carboxypeptidase D receptor binding site, the peptide binds hepatocytes and subsequently blocks DHBV infection [Urban and Gripon, 2002].

Peptide ligands that bind to the core antigen of hepatitis B virus (HBcAg) were selected from a random hexapeptide library displayed on filamentous phage. *In vitro*, one of them inhibited the interaction between HBcAg and the pre-S region of the L polypeptide, which is critical for virus morphogenesis. The result suggested that this peptide and the related small molecules might inhibit viral assembly [Dyson and Murray, 1995]. Further study confirmed that the interaction of L-HBsAg with core particles was critical for HBV assembly, and demonstrated in principle its disruption *in vivo* by small molecules [Bottcher *et al.*, 1998]. It was shown that two distinct segments of the hepatitis B virus surface antigen contribute synergistically to its association with the viral core particles [Tan *et al.*, 1999]. In binding assays *in vitro*, it was found that empty HBV core particles bound synthetic peptides corresponding to HBV envelope protein domains with the same affinity as did HBV DNA-containing core particles [Hourioux *et al.*, 2000]. Watts *et al.* studied the morphogenic properties of the peptide STLPETTVV, which could influence the HBV capsid protein assembly. It was suggested that linker peptides were attached to the capsid inner surface as hinged struts, forming a mobile array, an arrangement with implications for morphogenesis and the management of encapsidated nucleic acid [Watts *et al.*, 2002]. A suitable vector for the delivery of these peptides deserves to be found, and their antiviral efficacy should be evaluated *in vivo*.

2.1.7 Chimeric Core Protein

The restriction of HBV genome replication to the nucleocapsid makes this nucleoprotein particle an attractive target for intervention. Dominant negative (DN) core protein variants have been shown to interfere with nucleocapsid assembly. In animal model systems, transient expression of the DHBV molecular equivalent of the WHV and HBV DN constructs inhibited wild-type (WT) DHBV replication by 95% [Scaglioni *et al.*, 1996]. Von *et al.* [von *et al.*, 1996; 1999] fused DHBV Pol, DHBV S, lacZ and GFP, respectively, to the carboxyl terminus of the DHBV core protein to yield DN mutants that inhibit viral replication in the avian hepatoma cell line LMH. Core-Pol and core-S, but not core-lacZ or core-GFP, markedly interfered with RNA pregenome packing, while the DN core-GFP fusion protein formed mixed particles with WT core protein and interfered with reverse transcription of the viral pregenome. The result suggested that DN DHBV core proteins could target at least 2 steps within the viral life cycle, packaging of the viral pregenome and reverse transcription within mixed particles [von *et al.*, 1999]. More recent report showed that recruitment of core protein to the DHBV preassembly complex occurs in a cis-preferential manner. This mechanism may account for the leak of DN DHBV core protein mutants targeting reverse transcription [Von *et al.*, 2002].

A conceptually more powerful approach is capsid-targeted viral inactivation, which exploits a viral capsid protein or other virion-associated protein as a carrier to bring a degradative enzyme specifically into virus particles

[Natsoulis and Boeke 1991]. Beterams and Nassal [2001] found that C proximal fusion to the HBV capsid protein of the Ca^{2+}-dependent nuclease (SN) yields a chimeric protein. In HBV co-transfected human hepatoma cell, less than 1 coreSN protein per 10 WT capsid protein subunits reduced titers of enveloped DNA containing virions by more than 95%. Furthermore, no evidence was found that coreSN is cytotoxic. The calcium signaling, involved in stimulation of transcription and viral DNA replication [Bouchard *et al.*, 2001], initiates the antiviral action of the coreSN in the infected liver cell [Beterams and Nassal, 2001].

As intracellular immunogens, chimeric core proteins may induce cytotoxic T cells. It should be clarified whether their induction would soon abolish their antiviral efficacy or, by contrast, would further contribute to virus elimination by concomitantly inducing a response against WT core protein in the infected cells.

2.1.8 Single Chain Antibody

Several published reports have demonstrated that antibody genes can be expressed inside cells where the corresponding antibody fragments bind to their targets with high affinity and thus efficiently interfered with the function of cellular targets [Marasco *et al.*, 1993; Mhashilkar *et al.*, 1995, 1997; Cattaneo *et al.*, 1999]. A study using a cloned single chain Fv (sFv) fragment directed against HBsAg showed that this antibody fragment could reduce extracellular HBsAg levels by a mean of 85%. Confocal microscopy studies confirmed the intracellular expression and colocalization of the sFv and HBsAg [zu *et al.*, 1999]. A man-made antibody anti-HBc sFv could also inhibit viral replication intracellularly by forming sFv-HBc complex and interfering with the function of HBc [Yamamoto *et al.*, 1999]. Using the purified Pol protein to raise monoclonal antibodies (Mabs), Putlitz *et al.* generated six Mabs directly against HBV Pol protein, of which a Mab specific for the Pol terminal protein region appeared to inhibit Pol function in the *in vitro* priming assay. This represents an important first step towards the further exploration of the intracellular antibody strategy against HBV [zu *et al.*, 1999]. It remains to demonstrate their capacity for inhibiting viral replication *in vitro* and to find convenient ways for gene delivery *in vivo*.

2.1.9 Alpha-Glucosidase Inhibitors

One function of N-linked glycans is to assist in the folding of glycoproteins by mediating interactions of the lectin-like chaperone proteins calnexin and calreticulin with nascent glycoproteins. These interactions can be prevented with inhibitors of the alpha-glucosidases, such as N-butyl-deoxynojirimycin (NB-DNJ) and N-nonyl-DNJ (NN-DNJ), and caused some proteins to misfold and retain within the endoplasmic reticulum (ER). Evidence was given that M protein of HBV folded via a calnexin-dependant pathway [Werr and Prange, 1998]. The presence of NB-DNJ virions and the M protein were retained surprisingly [Mehta *et al.*, 1997], and proper intracellular routing of HBV glycoproteins was disrupted in cells where ER glucosidase was inhibited [Lu *et al.*, 1997]. In a woodchuck model of chronic HBV infection, the NN-DNJ-induced misfolding of HBV enveloped glycoproteins prevented the formation and secretion of infectious enveloped virus. This provided the first evidence that glucosidase inhibitors could be used *in vivo* and had anti-viral effects [Block *et al.*, 1998]. Further studies showed that NN-DNJ retained antiviral activity at concentrations that had no significant impact on ER glucosidase function. In addition, N-nonyl-deoxy-galactojirimycin (N-nonyl-DGJ), an alkyl derivative of galactose with no impact on glycoprocessing, retained anti-HBV activity. These results suggested that NN-DNJ possesses an antiviral activity attributable to a function other than an impact on glycoprocessing [Mehta *et al.*, 2001]. Therefore the mechanism of the alpha-glucosidase inhibitor action should be further elucidated to facilitate the application of these antiviral agents. They have already been demonstrated to have an antiviral efficacy against several viruses [van *et al.*, 1996; Zitzmann *et al.*, 1999; Wu *et al.*, 2002].

2.2 Immune Modulatory Strategies

2.2.1 Cytokines

Since systemic application of cytokines is associated with severe side effects, researches on targeted delivery or endogenetic expression through a gene therapy approach, have been prompted. Eto and Takahashi prepared an asialoglycoprotein (ASGP) receptor-directed interferon and compared its antiviral effects with that of conventional natural human IFNs. Their study demonstrated that directing IFN to ASGP receptor facilitated its signaling in the liver and augmented its antiviral effect [Eto *et al.*, 1999]. Man-made Anti-HBsAg interferon fusion proteins displaying both IFN activity and HBsAg have prompted an alternative way of making a targeting drug for hepatitis B [Tong *et al.*, 2001; Xia *et al.*, 2002]. Local production should provide IFN more efficient and better tolerant [Aurisicchio *et al.*, 2000]. Protzer *et al.* constructed a recombinant DHBV carrying the duck homolog of IFN-α, and superinfected DHBV-positive hepatocytes with rDHBV-IFN *in vivo*. DHBV-production decreased relatively to untreated controls, in a dose-dependent fashion, comparable to the maximal effect observed in the treatment with the IFN protein. No change in DHBV progeny production was seen on superinfection with rDHBV-GFP, indicating that the transduced IFN gene caused inhibition [Protzer *et al.*, 1999]. In an acute hepatitis model, the hepatic damage by mouse coronavirus MHV-3 infection was reduced by help-dependent adenovirus HD-IFN vector expressing mIFN-α 2. Challenged with ConA, the HD-IFN injected mice were protected, as HD-IFN exhibited a protective effect against liver injury even at doses that do not yield circulating mIFN-α 2 level [Aurisicchio *et al.*, 2000]. Recently, evidence has been produced that enhanced interferon-stimulated gene factor-3α (p48) expression increases IFN-α-induced suppression of HBV RNA significantly, in an experiment based on human hepatoma cells [Rang and Will, 2001]. This indicates that in order to optimize the IFN-α effect, interferon-stimulated response like element (ISRE) and the interferon-stimulated gene factor (ISGF) may be taken into account. Furthermore, ISGF was found to bind to ISRE-like sequence identified in the linker regions located between the heptameric tet operator sequence, resulting in IFN-α-mediated tet promoter stimulation activity. The data imply that the tet promoter-based expression system can be rendered non-responsive to IFN-α by mutagenesis of the ISREs, and this may be

essential when considering gene therapy *in vivo* [Rang and Will, 2000].

Other immunomodulatory cytokines, such as IL-12, IFN-α, or tumor necrosis factor-α, have potently suppressed HBV replication in an HBV transgenic mouse model [Guidotti *et al*., 1996; Cavanaugh *et al*., 1997], whereas IL-12 and the Th1 cytokines IFN-α and IL-2 seem to play an important role for viral clearance in chronically infected patients [Rossol *et al*., 1997, Guidotti *et al*., 1999]. To apply IL-12 genes in gene therapy, a pIL-12 vector was constructed that contained two cytomegalovirus (CMV) promoters to drive the expression of p35 and p40 subunits, respectively. In addition, a pscIL-12 vector was designed with a linker to fuse p35 cDNA with p40 cDNA to producing a single-chain IL-12 protein. The data suggested that the vectors could produce bioactive heterodimeric and single-chain murine IL-12. Furthermore, *in vivo* functional studies also demonstrated that mice co-immunized with a pS vector expressing the major envelope protein of HBV and pIL-12 or pscIL-12 elicited higher levels of IgG2a anti-HBs antibody and of Th1-related cytokine. The success in using a single promoter to express single-chain IL-12 indicated that pscIL-12 should be useful in future applications for gene therapy [Lee *et al*., 1998].

2.3 HBV Antigen

DNA-mediated immunization has been shown to be an effective way to induce both homoral and cell-mediated immune responses against many different HBV antigens. First, the envelope protein of HBV, i.e. HBsAg, was chosen as a model for DNA vaccination, as it carried the major antigenic determinant of the virus [Mancini *et al*., 1996; Michel *et al*., 1995, 2001; Geissler *et al*., 1998; Davis *et al*., 1996]. Using the HBsAg transgenic mouse as a model, Mancini *et al*. studied immunization mediated by the S and pre-S2 domains of the gene encoding the HBV envelope protein, and found that the induced immune response resulted in the complete clearance of circulating HBsAg and in the long-term control of transgene expression in hepatocytes. The study showed that T cells were responsible for the down-regulation of HBV mRNA in the liver. This was the first demonstration of potential immunotherapeutic application of DNA-mediated immunization against an infectious disease that raises the possibility of designing more effective ways of treating HBV chronic carriers [Mancini *et al*., 1996]. Recently, they explored the ability of CpG-containing oligodeoxynucleotides combined with recombinant HBsAg to induce Th1 responses in the same model, and suggested that DNA motifs containing unmethylated CpG dinucleotides within the context of certain flanking sequences enhanced both innate and antigen-specific immune responses, due in part to the enhanced production of Th1-type cytokines [Malanchere-Bres *et al*., 2001]. In addition, with HBV-transgenic mice, it was demonstrated that the activation of dendritic cells following injection with vaccine containing HBsAg is the vital factor underlying the therapeutic potentiality of vaccine therapy in HBV carrier [Akbar *et al*., 1999]. As the HBV core antigen (HBcAg) and e antigen (eAg) are highly conserved between HBV subtypes, they are attractive targets for an immune-based therapeutic treatment [Sallberg *et al*., 1997,1998; Townsend *et al*., 1997]. Townsend *et al*. showed that intramuscular injections of a novel recombinant retroviral vector expressing an HBcAg-neomycin phosphotransferase II (HBc-NEO) fusion protein induced HBc/eAg-specific antibodies and CD4+ and CD8+ T cell responses in mice and rhesus monkeys [Townsend *et al*., 1997]. When three chronically infected chimpanzees were immunized with nonreplicating retroviral vector particles expressing the HBc-NEO fusion protein, one exhibited a traditional seroconversion, while the other two showed transient ALT flares and a significant decrease in the serum HBV DNA levels [Sallberg *et al*., 1998]. Recently, CTL responses against HBV polymerase were assayed. Immunized mice exhibited substantial polymerase-specific CTL responses. This is the first study to demonstrate the generation of a CTL response to HBV pol by immunization. The next task is to investigate the response either in infection models or in transgenic mice that fully replicate HBV. In addition, the validation of HBV polymerase as a target for DNA-based immunization requires further investigation of its potential toxic effects when expressed at high levels in cells [zu *et al*., 2000].

The expression of cytokine or a costimulatory protein and HBV antigen in the same cells *in vivo* induces stronger cellular and humoral immune response than expression of the antigen alone as demonstrated by several studies of IL-2 [Geissler *et al*., 1998; Chow *et al*., 1997], GM-CSF [Geissler *et al*., 1998], IL-12 [Lee *et al*., 1998], B7-1 [He *et al*., 1996], and B7-2 [Zhou *et al*., 2001]. This could be a novel strategy for the development of therapeutic vaccines against infectious agents. Conventional vaccine combined with CpG oligodeoxynucleotides motifs [Malanchere-Bres *et al*., 2001], or vaccine with new peptide, which can elicit priming of antigen-specific cytotoxic T lymphocytes [Meng *et al*., 2001], may also be promising therapeutic approaches and deserve confirmation in further studies. Furthermore, these immunomodulatory agents should be more useful when combined with drugs that are capable of blocking viral replication.

3. DELIVERY METHODS

3.1 Viral Vectors

Delivery of genes for stable gene expression requires the use of an efficient gene delivery system, such as replication defective viral vectors. Currently, mouse retroviral vectors have widely been used in gene therapy, as they provide efficient transduction of a wide range of cell types and the genes are stably integrated and expressed in the host cell. To achieve high transgenic expression in the liver, various murine retoviral long terminal repeats (LTRs) or leader sequences were compared, and higher gene expression was observed by the FMEV-type vector, which contained the spleen focus-forming virus (SFFVp) LTR and the mouse embryonic stem cell virus (MESV) leader, than by the Moloney murine leukemia virus (MoMLV)-based vector [Ohnishi *et al*., 2002]. To expedite analysis in anti-HBV gene therapy, retroviral vector was used to transfer antisense [Tung and Bowen 1998; Ji *et al*., 1997]. It is safe to utilize retroviral vector encoding HBcAg to immunize chimpanzees and stimulate immune responses in HBV chronic carrier

chimpanzees; demonstrating retroviral vector immunization may be beneficial in the immuno-gene therapy for chronic HBV infection [Sallberg *et al.*, 1998]. However, conventional retroviral vectors may not be an ideal vector system to deliver genes into hepatocytes *in vivo* because the majority of liver cells are not dividing. Furthermore, retroviral vectors can be generated in only limited quantities and have a broad host-range that does not conduce to the hepatocyte tropism required for HBV therapy. This problem has been overcome by deriving vectors from lentiviruses (a class of retroviruses) that have the ability to infect both dividing and nondividing cells. The lentiviral vectors are derived from human immunodeficiency virus type 1 (HIV-1) [Naldini *et al.*, 1996]. More recently Sung *et al.* have developed a system for producing murine leukemia virus (MLV) pseudotyped with large (L) and small (S) HBsAg for targeting primary human hepatocytes. The MLV (HBV) pseudotype virus remains the strict hepatotropsim of the natural HBV, and does not infect any of the established tissue culture cell lines. The presence of both L and S forms enhanced the surface expression of HBsAg and thus increased virus production. This virus offers a potential liver-specific targeting system for gene therapy [Sung and Lai, 2002].

Gene therapy is not a low risk/benefit approach. Safety is always of paramount importance for clinical gene therapy. Humans have an immune system to fight off the virus, and our attempts to deliver genes in viral vectors have been confronted by these host responses [Xu *et al.*, 2003]. The issues and assays needed to ensure patient safety with this new vector system are still being defined [Podsakoff, 2001]. It has been suggested that the possibility of inadvertent transfer of a mobilized vector to a partner could have potentially serious consequences. Vector mobilization at any level is problematic because it might lead to unwanted recombination events, which could adversely affect a trial subject. More thorough characterization of potential outcomes is necessary before it can be applied in the clinic [Podsakoff, 2001]. The clinical trial of gene therapy for X-linked severe combined immune deficiency (SCID) performed by the French investigators still has been seven of 10 subjects in good health with their immune systems restored by the gene treatment. These findings highlight the potential of gene therapy to correct this otherwise fatal immune disorder without complications, such as graft rejection, that may be seen when hematopoietic stem cells from another donor are used in a "standard" bone marrow transplant approach. However, the leukemia developed in two of 10 infants treated for SCID by gene therapy was observed [Geiger, 2003]. This event is directly related to the retroviral-mediated insertion of the gene products. A key scientific question to be explored is why this problem has only been seen so far in this study of infants treated for XSCID, but not in any of the other clinical trial using retroviral vectors targeted to hematopoietic stem cells or any other trial of gene therapy [Geiger, 2003]. The extensive studies animal models of cancer revealed that genome-wide retroviral insertional tagging of genes involved in cancer in Cdkn2a-deficient mice [Lund *et al.*, 2002] and the new genes involved in cancer were also identified by retroviral tagging [Suzuki *et al.*, 2002]. Therefore, further assessment of the risk to patients must be completed prior to initiation of any new clinical trial.

Adenoviruses (Ad) are hepatotropic when injected intravenously and can be generated at a very high titer, but the use of adenoviral vectors has been limited due to host immune responses against the vector and/or transgene and vector toxicity [Yang *et al.*, 1994]. To decrease side effects associated with viral gene expression, further attenuating of viral gene expression by eliminating viral genes has been attempted. Significantly diminished vector toxicity was obtained in mice treated with E1/E2a/E3/E4-deficient Ad vectors. However, the duration of transgene expression mediated by this vector was reduced [Andrews *et al.*, 2001]. Hodges *et al.* generated Ad vectors with 100K gene deleted, and demonstrated that injection of an [E1-, 100K-]Ad vector *in vivo* is correlated with significantly decreased hepatotoxicity, as well as prolonged vector persistence [Hodges *et al.*, 2001]. These results confirmed that adenoviral vectors with all viral coding sequences deleted offer the prospects of decreased host immune responses to viral proteins, decreased cellular toxicity of viral proteins, and increased capacity to accommodate large regulatory DNA regions [Schiedner *et al.*, 1998]. A similar effect occurs when using an Ad-based vector, the encapsidated adenovirus mini-chromosome (EAM) from which all of the viral genes have been deleted [Kumar-Singh, 1998]. Use of a liver-specific promoter also can reduce immune response to the transgene in adenoviral vectors [Pastore *et al.*, 1999]. The combination of a helper-dependent adenovirus vector and liver-specific promoter resulted in intrahepatic IFN-α expression, which protected the liver in acute hepatitis model [Aurisicchio *et al.*, 2000]. It was reported that episomal segregation of the adenovirus enhancer sequence by conditional genome rearrangement abrogated late viral gene expression. A recombinant adenovirus gene delivery system with the capability of undergoing growth phase-dependent site-specific recombination has been constructed. Because no helper virus is required to propagate these vectors, the problems of recombination with and contamination by helper virus are eliminated [Wang *et al.*, 2000]. Another report showed that inserting inverted repeats (IRs) into the E1 region of the Ad vector could mediate predictable genomic rearrangements, resulting in vector genomes devoid of all viral genes [Steinwaerder *et al.*, 1999].

Adeno-associated virus (AAV), a nonpathogenic, single-stranded DNA virus, can transduce both dividing and nondividing cells, and achieve long-term expression of therapeutic genes with no apparent adverse effects. Most importantly, several groups have documented the ability to deliver sustained liver-targeted transgene expression in an immunocompetent host for more than 1 year, and that the curative level of the gene product from one injection is sustained for long-term in the animal [Wang *et al.*, 2000; wang *et al.*, 1999; Jung *et al.*, 2001; Xu *et al.*, 2001]. Comparable results have not been achieved with any other vector to date. Additionally, a gene 'pill', i.e. an AAV vector administrated perorally, associated with highly efficient and stable gene expression, should render AAV vectors a palatable choice compared with current pharmacological treatment [During *et al.*, 1998; 2000; Xu *et al.*, 2003]. Recently, research has been conducted to reveal the

chromosomal effects of AAV vector integration. It was reported that integrated vector proviruses are associated with chromosomal deletions and other rearrangements [Miller *et al.*, 2002].

Hepatitis B virus with the distinct liver-targeted features is an attractive candidate as a vector for gene therapy of acquired liver diseases. Construction of a vector from HBV DNA has been attempted [Chaisomchit *et al.*, 1997]. Hanafusa *et al.* showed that HBV could carry 63 bp of extra DNA [Hanafusa *et al.*, 1999]. Four novel cir-acting elements were reported to be essential for the viral genome synthesis. According to this result, a recombinant HBV-GFP vector was generated, which can replicate as efficiently as that of the wildtype [Ryu and Lee 2001]. As a first step toward therapeutically useful hepadnavirus vectors, Protzer *et al.* constructed a recombinant DHBV carrying the duck homolog of IFN-α, which efficiently suppressed wild-type virus replication [Protzer *et al.*, 1999]. However, its potential use as a gene transfer system may be limited by its small capacity, due to the small size of the HBV genome.

3.2 Nonviral Vectors

Naked DNA acts as a simple, safe and viable alternative for gene therapy. The use of plasmid vectors expressing the HBV antigens alone, or coexpressing with cytokine, for transfection of muscle fibers has been demonstrated to be a potential immunotherapeutic application against HBV infection [Mancini *et al.*, 1996; Geissler *et al.*, 1998; Chow *et al.*, 1997]. HBsAg-specific humoral or cell-mediated responses are not induced in mice when the muscle-specific human muscle creatine kinase promoter is used in plasmid DNA vaccine. This result suggested use of a tissue-specific promoter that does not drive expression in antigen-presenting cells [Weeratna *et al.*, 2001]. Several approaches aiming at enhancing nonviral transgene delivery have been investigated. One approach is to pulse electrical fields (electroporation or EP) after naked gene injection [Glasspool-Malone *et al.*, 2000]. These advances create new opportunities for nucleic acid vaccine development. Evidence showed that electroporation enhanced that the delivery of plasmid DNA encoding IL-12 to skin [Heller *et al.*, 2001] or to skeletal [Lucas and Heller 2001] muscle. The molecule was efficiently delivered, and one of the molecules that induced (IFN-gamma) was also measured systemically in this successful delivery to skeletal muscle [Lucas *et al.*, 2001]. The use of *in vivo* electroporation in immunotherapy protocols deserves further examination. Another approach is to combine plasmid DNA to other compounds. Poloxamers [Lemieux *et al.*, 2000], aurintricarboxylic acid [Glasspool-Malone *et al.*, 2000], nuclear localization signal (NLS) peptides [Schirmbeck *et al.*, 2001] and some other polymers [Prokop *et al.*, 2002] have been demonstrated to be good candidates for enhancing the efficiency of gene transfer.

When liposome, one of the most popular gene transfer systems *in vitro* in the laboratory [Felgner and Ringold, 1989], was used *in vivo* as liver-directed gene transfer, it was observed that its transfection efficiency depended on its route of administration [Otsuka *et al.*, 2000; Hirano *et al.*, 1998; Mohr *et al.*, 2001], and the efficiency can be increased by liver resection, ischemia or transplantation performed before DNA injection [Otsuka *et al.*, 2000]. Liposome can deliver not only the genes, but also the drugs, to the liver. To prevent degradation of antisense molecules *in vivo*, Soni *et al.* [Soni *et al.*, 1998] showed that liposomes could increase the hepatic delivery and antiviral efficacy of phosphorothioate antisense oligodeoxynucleotides (PS-ODN).

Many attempts for receptor-mediated liver-targeted delivery have been performed successfully. Since asyaloglicoprotein receptor is specific for hepatocytes, DNA-protein complexes using asyaloglicoproteins [Nakazono *et al.*, 1996; Wu and Wu 1992] or protein conjugates, consisting of N-acetyl-glucosamine-modified bovine serum albumin, streptavidin and Poly-L-lysine [Madon and Blum 1996], have been shown to be effective at delivering antisense DNA to suppress HBV gene expression *in vitro*, and this receptor-mediated endocytosis showed no host toxicity [Wu *et al.*, 2001]. An N-glycosylated human IFN-α was generated, which exhibited a significantly higher antiviral effect than conventional IFN-α *in vivo* [Boni *et al.*, 2001]. Complexed with N-acetylglucosamine, a recombinant human adenovirus, which does not naturally infect avian cells, it allowed highly efficient and specific gene transfer into the liver of ducks *in vivo*. This result represents a novel approach to gene therapy for inherited and acquired liver diseases [Thoma *et al.*, 2000]. The functional ability of synthetic galactose polymer ligand was evaluated and poly-(N-p-vinylbenzyl-O-beta- D-galactopyranosyl- [1-4]-D-gluconamide) (PVLA) was found to exhibit higher affinity with hepatocytes than natural ligands [Watanabe *et al.*, 2000]. Oral administration of cholesterol-modified phosphorothioate antisense oligonucleotides (Chol-S-ODNs) has also been shown to target the liver, and has been suggested as a practical method for the long-term treatment of chronic diseases [Okamoto *et al.*, 1999]. In addition, *in vivo* gene delivery to the liver may achieve success by some other nonviral vectors, such as reconstituted remnants of chylomicron, the first nonviral vector to resemble a natural lipoprotein carrier [Hara *et al.*, 1997]. Linear polyethylenmine (lPEI)-mediated transfer was also shown to be a good delivery method in the duck model [Robaczewska *et al.*, 2001].

3.3 Liver Transplantation with Combination Therapy

More recently, a combination of hepatitis B immune globulin (HBIG) and lamivudine has been shown to prevent HBV recurrence effectively in patient post-orthotopic liver transplantation for hepatits B virus infection. Recent studies have revealed that in the combination therapy group no patient redeveloped serum HBsAg or HBV DNA during mean follow-up of 459 and 416 days, respectively. In the monotherapy group, there was a reappearance of HBaAg in the serum of 3 patients (25%) during a mean follow-up of 663 days [Han *et al.*, 2000]. Combination prophylaxis with HBIG and lamivudine is highly effective in preventing recurrent HBV, may protect against the emergence of resistant mutants, and is significantly more cost-effective than HBIG monotherapy with its associated rate of recurrent HBV. Famciclovir and lamivudine also reduced viral replication in patients with recurrent hepatitis B virus infection after orthotopic liver transplantation [Tillmann *et*

al., 1999]. Since the treatment break through is frequent for this specific group of patients, and there are no significant adverse side effects, the use of liver transplantation with combination therapy should be explored.

4. ANIMAL MODELS

The infection of ducks [Mason *et al.*, 1980], woodchucks [Summers *et al.*, 1978], and squirrels [Marion *et al.*, 1980] with their respective animal hepatitis viruses has been an important step in gaining much of our knowledge of HBV infection. The similarity between HBV and the closely related DHBV makes the latter a convenient model for the study of molecular mechanisms of HBV replication and neutralization and for the screening of antiviral agents [Chassot *et al.*, 1993; Mi *et al.*, 1995]. However, DHBV is not typically associated with liver disease. Woodchucks chronically infected with WHV develop progressively severe hepatitis and hepatocellular carcinoma similar to those associated with HBV infection in humans. Chronic WHV carrier woodchucks have become a valuable animal model for preclinical evaluation of anti viral therapy for HBV infection, providing useful pharmacokinetic and pharmacodynamic result in a relevant animal disease model. This model also has significant potential for the preclinical assessment of antiviral drug toxicity [Tennant and Gerin 2001]. However, due to the high level of divergence between HBV and these viruses and the considerable metabolic differences between their hosts and humans, the utility of these models is often limited.

The best animal model to date for infections with human HBV is the chimpanzee. However, because chimpanzees are large-sized and highly intelligent animals, and also an endangered species, their use is reserved for essential experiments.

Mice are not susceptible to HBV infection, but a number of lineages of mice have so far been developed to carry one or more HBV transgenes. Guidotti *et al.* successfully generated transgenic mice that replicated high levels of human hepatitis B virus in clinically important target organs of the liver and kidney. Sera from these mice contain a high titer of viral DNA approaching to that found in the natural chronic human infection [Guidotti *et al.*, 1995]. This model has been demonstrated to be a valuable therapeutic model for HBV [Morrey *et al.*, 1999; Morrey *et al.*, 1999]. However, since the transgenic mouse are tolerant of HBV antigens, there are limitations to the use of transgenic mouse models in the study of the mechanisms by which the anti-HBV cellular immune response leads to liver disease. Recently, an HBV transgenic severe combined immunodeficiency (SCID) mouse was created. These mice consistently supported HBV gene expression and replication. After adoptive transfer of syngeneic, unprimed splenocytes, these mice reproducibly cleared virus markers from the liver and serum, and developed chronic hepatitis. This unique model provided an opportunity to elucidate the pathogenesis of chronic liver disease and to evaluate new approaches aimed at both the virus and the disease [Larkin *et al.*, 1999]. A recent study has provided the first evidence that adenovirus-mediated genome transfer initiated efficient hepatitis B virus replication in cultured liver cells and in the experimental animals from an extrachromosomal template. Allowing the development of small-animal systems of hepatitis B virus infection, and facilitating the study of the pathogenicity of wild type and mutant viruses, virus-host interaction, and new therapeutic approaches [Sprinzl *et al.*, 2001].

An alternative way of developing an HBV-carrying mouse model can be achieved by transplanting human hepatocytes. The hepatitis B virus-trimera mouse was created by the implantation of *ex vivo* HBV-infected liver fragments into lethally irradiated mice, radioprotected with SCID mouse bone marrow cells. Viremia attained a peak between days 18 and 25, while HBV infection is observed in 85% of the transplanted animals 1 month post-implantation [Ilan *et al.*, 1999]. Compared with the trimera model, a newly reported xenotransplant model exhibited some advantages. These mice were susceptible to HBV infection and completion of the viral life cycle. Furthermore, they can be super-infected with HDV. This study demonstrates that human hepatocytes can be engrafted on a long-term basis in mice, and serve as a model for human diseases such as HBV and HDV infection. This model therefore offers an important opportunity of studying multiple aspects of human hepatitis viral infection, and may enhance studies of human liver diseases [Ohashi *et al.*, 2000]. Transplanting human hepatocytes and inoculating HBV generated a model of human hepatitis B infection (HBV) in immunocompetent rats after birth. [Wu *et al.*, 2001]. Other researches have proved that normal human hepatocytes can integrate into the mouse hepatic parenchyma, undergo multiple cell divisions, and remain permissive for a human hepatotropic virus in a xenogenic liver [Dandri *et al.*, 2001].

In addition, the attempts to develop small primate models with hepadnaviruses closely related to HBV have provided a potential animal model for HBV research, e.g., the woolly monkey (*Lagothrix lagotricha*), from which a hepadnavirus (WMHBV) has been isolated [Lanford *et al.*, 1998] and is under investigation [Kock *et al.*, 2001]. The molecular biology of WMHBV should be fully understood before the WMHBV/woolly monkey model system is applied in anti-HBV research.

Better animal models of HBV infections are still extremely needed to test antiviral strategies for eliminating chronic liver disease. It was reported that natural human HBV could enter Wistar rat liver cells through intravenous injection efficiently, and express for a long period [Wang *et al.*, 1996]. This implies the possibility of a rat model infected with HBV.

CONCLUSION

Simultaneously with the increase in our knowledge of HBV molecular biology, especially of the replication mechanisms, a number of virus-specific proteins and nucleotide analogs, such as adfovir, or processes, have also been identified as targets for transgene intervention. Only some of these have been addressed here. Clinical trials indicate that different types of combined therapy may have to be tailor-made for chronic HBV infection. More targets identified, more alternatives there are. Studies in gene therapy for other viruses, e.g. HIV, have been very helpful into anti-HBV research. The effectiveness of combination

genetic therapy has been reported in inhibiting HIV-1 [Strayer *et al.*, 2002; Lisziewicz *et al.*, 2000]. But there are limited delivery systems for synchronous expression of multigenes. Development and testing of combination anti-HBV genetic therapies require both transgenes that effectively inhibit HBV individually or cooperatively, and a vector that delivers these transgenes at high efficiency. Keeping HBV out of the cells and depriving the viruses of infectious ability may be realized by the studies on the early events of the viral life cycle. Although cloning of the DHBV receptor may aid in the identification of the HBV human counterpart [Tong *et al.*, 1999], there are still many unanswered questions in cell culture system. No permanent cell lines are permissive to HBV infection, and primary human hepatocytes are not easily available for *in vitro* infection studies. More importantly, an animal model mimicking natural HBV infection is needed to understand the mechanisms behind the process from acute to chronic states and to optimize the protocols of immunotherapy and/or antiviral therapy. Nevertheless, molecular therapy approaches, because of the promise they show, look set to be increasingly applied in clinical treatment in next few years.

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